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Pharmacological interventions in the Wnt pathway: Inhibition of Wnt secretion versus disrupting the protein-protein interfaces of nuclear factors

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Abstract

Mutations in components of the Wnt pathways are a frequent cause of many human diseases, particularly cancer. Despite the fact that a causative link between aberrant Wnt signaling and many types of human cancers was established more than a decade ago, no Wnt signaling inhibitors have made it into the clinic so far. One reason for this is that no pathway-specific kinase is known. Additionally targeting the protein-protein interactions needed to transduce the signal has not met with success so far. Complicating the search for and use of inhibitors is the complexity of the cascades triggered by the Wnts and their paramount biological importance. Wnt/β-catenin signaling is involved in virtually all aspects of embryonic development and in the control of the homeostasis of adult tissues.

Encouragingly however, in recent years first successes with Wnt-pathway inhibitors have been reported in mouse models of disease. In this review we summarize possible roads to follow during the quest to pharmacologically modulate the Wnt signaling pathway in cancer.

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List of abbreviations

Frizzled	FZD
Low density lipoprotein 5	LRP5
Porcupine	PORCN
Wntless	WLS
Secreted Frizzled Related Proteins	SFRPs
Dickkopf	DKK
Transforming Growth Factor Beta	TGF-β
Adenomatous Polyposis Coli	APC
Glycogen Synthase Kinase	GSK3β
Casein Kinase	CK1
Pygopus	Pygo
Bcl9 and Bcl9l	Bcl9/9l

Introduction

Wnts activate diverse signaling cascades

Mammalian genomes encode for 19 different Wnt molecules, which can bind to 10 different Frizzled (FZD) receptors (Koike et al., 1999)(Langton et al., 2016). Frizzleds belong to the family of seven-pass transmembrane G-protein coupled receptors. When bound by Wnt proteins on their extracellular cysteine rich domain, they activate the cytosolic protein Disheveled to transduce the signal inside the cell (reviewed in (Dijksterhuis et al., 2014). Several independent Wnt signaling cascades are activated in response to Wnts binding to their cognate receptors. The best studied, and perhaps the most important is the β-catenin dependent signaling cascade, mediated by β-catenin (Figure 1a,b). The β-catenin dependent cascade is of preeminent importance for development and tissue homeostasis. When deregulated it causes the initiation and progression of a myriad of different tumor types. Besides the β-catenin-mediated cascade, there are other β-catenin independent outputs, such as the planar cell polarity and the Wnt/Ca^{2+} signaling. The nature of the pathway transduced depends on the receptors/co-receptors present (He et al., 1997) (van Amerongen et al., 2008). To transduce the β-catenin dependent signal, Frizzled proteins bind the co-receptors Low density lipoprotein 5 (LRP5) or LRP6. Why in each specific context a particular Wnt/receptor combination activates one cascade or another is not entirely clear. However, some Wnts are thought to be preferentially β-catenin dependent (e.g. Wnt3a) or independent (e.g. Wnt5a). Wnt5a normally binds to Frizzled and Ror/Ryk instead of Lrp5/6 and activates, amongst others, JNK signaling (Yamanaka, 2002). β-catenin independent signaling is often associated with the regulation of cell adhesion, migration and polarity (reviewed in (Veeman et al., 2003)). Furthermore, it is also thought to repress β-catenin dependent signaling (Yuzugullu et al., 2009). The β-catenin independent cascade has received increasing attention in recent years due to its role in melanoma formation and metastasis (Chien et al., 2009) (Weeraratna et al., 2002).

While the search for therapeutic targets has long focused on the transduction of the signal in the receiving cell, it is increasingly evident that an alternative strategy to modulate the Wnt signaling cascade is at the level of the ligands, for example inhibiting their secretion.

Wnt secretion is dependent on Porcupine and Wntless

To be fully active, Wnts must undergo glycosylation and lipid modification (Figure 2). Whereas Wnt glycosylation enhances but is not essential for secretion and signaling, the lipid modifications are necessary for both functions. Wnts are acylated on two conserved residues, corresponding to cysteine 77 and serine 209 in mouse Wnt3a (Harterink and Korswagen, 2012). The enzyme responsible for these lipid modifications is the O-Acyl-transferase Porcupine (PORCN). This is demonstrated by the fact that the genetic loss of PORCN, or the impairment of its activity, leads to retention of Wnt molecules in the endoplasmic reticulum (ER). The acylation of *Drosophila* Wnts in position Ser209 (or the mammalian homolog position) is required for the interaction of Wnts with Wntless (Wls), which is another protein critical for Wnt secretion (Herr and Basler, 2012). Wls is a multipass transmembrane protein that is absolutely required for the secretion of all Wnts (Bänziger et al., 2006)(Bartscherer et al., 2006). The puzzle of how Wls promotes Wnt secretion remains unresolved; however, many pieces have already been put together. These include the unearthing of the role of the retromer complex in the retrieval of Wls, which establishes a trafficking loop from the ER to the plasma membrane via the Golgi (Herr et al., 2012). While our understanding of Wls function is not sufficient to generate small molecule inhibitors, for the enzyme PORCN suitable inhibitors have been discovered. Porcupine is an attractive target because it seems to be exclusively required for Wnt secretion. Moreover, we have also found that PORCN, which is the sole enzyme known to be specific to the Wnt cascade, is upregulated in murine cancer models. Additionally, elevated PORCN expression is an indicator for bad prognosis in head and neck squamous cell carcinomas (the cancer genome atlas, unpublished observations by Dario Zimmerli).

Wnt-signaling initiation by Wnt-Frizzled interaction is highly regulated

Wnt signaling transduction is tightly regulated at the level of the ligand-receptor interaction. This is achieved by titration of the ligands and/or of the receptors.

Ligand availability can be modulated by the production of secreted frizzled related proteins (SFRPs). SFRPs are secreted molecules with no direct signaling activity, but they possess a Wnt-binding domain via which they sequester extracellular Wnts (Leyns et al., 1997)(Wang

et al., 1997). Another way to modulate Wnt signaling is to alter the level and/or availability of the receptors or co-receptors. The four secreted Dickkopf (DKK) proteins are a well-studied class of molecules that act in this way. In the Wnt cascade, DKKs act by binding to the FZD co-receptors LRP5/6, thereby inhibiting the binding of the Wnts (Mao et al., 2001). Three of the DKK proteins (DKK1, 2, and 4) appear to be specific for the Wnt pathway and act by binding to LRP5/6 (Mao et al., 2001). Interestingly, DKK2 and DKK4 can act as either activators or as repressors of the pathway, depending on the abundance of the co-factor KREMEN2 (Mao and Niehrs, 2003). In contrast to the three other members of the DKK family, DKK3 acts in the transforming growth factor beta (TGF-β) signaling cascade (Pinho and Niehrs, 2007)(Nakamura and Hackam, 2010). In addition to above mentioned mechanisms, there is a variety of other transmembrane or secreted inhibitors with various modes of action, such as WIF, WISE/SOST, CERBERUS, IGFBP, TIKI1, SHISA, WAIF1 and APCDD1 (reviewed in (Cruciat and Niehrs, 2013).

Besides Lrp proteins, there are other receptor-co-receptor pairs such as Ryk, which can enhance Wnt signaling (Lu et al., 2004). Additionally there are ancillary receptor complexes, which regulate the levels of available Wnt receptors. Most prominent among them are LGR4/5/6. Those proteins came to fame as Wnt target genes expressed in the intestine and were found to mark various stem cell populations (Barker et al., 2007). Later on, it was revealed that they greatly increase Wnt signal transduction when they are bound by the extracellular R-spondin. They act by inhibiting Frizzled ubiquitination and subsequent degradation by ZNRF3 and RNF43 (de Lau et al., 2014).

The diversity of mechanisms by which Wnt-signaling initiation by Wnt-Frizzled interaction is regulated is both a bane and a boon. There are many potential targets but their diversity also means that redundancy could affect the efficacy of any intervention.

β–catenin is the central scaffold transducing the β-catenin dependent Wnt signal

The central node of the β-catenin dependent pathway is β-catenin. β-catenin was discovered as a membrane-associated protein that binds E-cadherin (Kemler, 1993). Later it was found that it regulates Wnt-dependent transcription via the recruitment of different transcriptional cofactors to the regulatory regions of Wnt target genes. In a signaling “off state”, the so-

called ‘destruction complex’ (consisting of adenomatous polyposis coli (APC), Axin, and the kinases responsible for the phosphorylation of β-catenin - glycogen synthase kinase (GSK3β) and casein kinase (CK1)) marks cytosolic β-catenin for proteosomal degradation (Stamos and Weis, 2013). Upon pathway activation the rate limiting factor of the destruction complex, Axin, together with GSK3β, is recruited to the so-called Wnt signalosome - consisting of WNT/FZD/LRP and multimerized Dishevelled (Bilic et al., 2007). This destabilizes the destruction complex, leaving β-catenin free to accumulate and to translocate into the nucleus, where it binds to the transcription factors of the TCF/LEF family. Acting together with a plethora of N- and C- terminally binding transcriptional co-activators, β-catenin and TCF/LEF facilitate target gene expression.

In the pathway “off-state” TCFs are thought to silence target genes by recruiting co-repressors such as Groucho. These co-repressors are displaced by β-catenin and its cohort of transcriptional activators (Städeli et al., 2006; Mosimann et al., 2009) (Clevers, 2006)(Valenta et al., 2012).

Recently, it was discovered that even if β-catenin escapes degradation by the destruction complex, it can still be degraded by the proteasome unless rescued by Armless, a pathway component recently identified in *D. melanogaster*. Armless protects Arm/β-catenin from degradation by inhibiting the function of Ter94 in facilitating protein turn over (Reim et al., 2014). This discovery is interesting in light of this review, as it might represent a so far overlooked mechanism for therapeutic intervention.

The Wnt/β-catenin transcriptional pathway is executed by N- and C- terminal co-activators

β-catenin facilitates transcription by recruiting several N- and C- terminally binding co-activators.

The factors directly binding the N-terminus of β-catenin are Bcl9 and Bcl9l (the two mammalian paralogs of the Drosophila Legless), they in turn recruit Pygopus (Pygo1 and 2 in mammals). Bcl9/9l and Pygopus are thought to form a “chain of adaptors” extending from β-

catenin. The simple model arising from Drosophila is that Legless and Pygopus are essential for the Wnt transcriptional output (Kramps et al., 2002)(Thompson et al., 2002), (reviewed in (Mosimann et al., 2009) and (Valenta et al., 2012)). In mammals, while also required for a maximal Wnt output, the relative importance of Bcl9/9l and Pygopus seems to be context dependent. In the mouse loss of function mutations in these genes do not recapitulate loss of β-catenin dependent Wnt signaling (i.e. by mutations in β-catenin). For example β-catenin signaling mutants die at E6.5, whereas Bcl9/9l knockout (KO) animals die at E10.5, while Pygo KO animals survive at least to E13.5. Moreover, recent work has demonstrated that the Pygo-Bcl9 complex can also act independently of β-catenin (Cantù et al., 2014), (Cantù et al., 2017). We therefore speculate that the role of Bcl9 as well as Pygo is to act as boosters of the signal and facilitate transcription of specific target genes in a subset of cells with active Wnt signaling. As we describe later the context dependent requirement of the so-called N-terminal chain of adaptors for facilitating the Wnt transcriptional output presents an exciting therapeutic target.

Another series of cofactors bind to β-catenin's C-terminus. This ensemble of cofactors comprises a diverse group of proteins, which have a more general role in transcription initiation and progression – reviewed in (Mosimann et al., 2009)(MacDonald et al., 2009). Most prominent among them are p300 and CBP, members of the basal transcriptional machinery, which were thought to have redundant modes of action in transcriptional activation (Takemaru and Moon, 2000),(Hecht, 2000). However, recent studies suggest that although they are redundant in certain tissues, p300 and CBP can play decisive roles and determine the nature of the transduced Wnt transcriptional program: In lung fibrosis, the differential utilization of CBP or p300 seems to make the decision to execute alveolar repair or promote fibroproliferation associated with fibrosis (Gottardi and Königshoff, 2013), (Kahn, 2014).

Wnt signaling in cancer

Since Wnt signaling plays a role in nearly all developmental processes, it does not come as a surprise that it is also implicated in many cancers. There are several possibilities for a cancer cell to hijack this pathway. It can either inactivate/decrease the expression of an inhibitory component or activate/increase the expression of an activating factor. When, in 1991, mutations in the APC gene were discovered in 80% of colorectal cancers, efforts to find a drug acting on this protein were initiated, so far with limited success (Groden et al., 1991) (Powell et al., 1992). In addition to mutations in APC, which is a fundamental component of the β-catenin destruction complex, other Wnt pathway mutations have been found: rarely inactivating mutations in Axin and activating mutations in the gene encoding for β-catenin (10%). Whereas in colon cancer APC mutations and β-catenin are prevalent, in other cancer types, such as hepatocellular carcinoma, mutations in Axin predominate. Oncogenic *ctnnb* mutations occur in melanoma and in solid tumors such as thyroid tumors (Kahn, 2014)(Mazzoni and Fearon, 2014). The fact that in different tumors alternate Wnt signaling activating mutations occur means that alternate strategies may need to be employed in each case. This will be further discussed in the specific sections for the different targets.

As shown in colon cancers, the Wnt pathway is also activated in some tumors through epigenetic silencing of inhibitors of the cascade (Suzuki et al., 2004). Some of these epigenetic changes affect the secreted inhibitors that regulate transduction at the level of the Wnt pathway ligand-receptor interaction: Methylation of *SFRP* genes, for instance, has been reported in colon, breast, lung, prostate and other cancers (Caldwell et al., 2004)(Suzuki et al., 2004)(Fukui et al., 2005). Mutations in inhibitory factors like ZNRF43 and RNF43 have also been reported. These proteins act as negative regulators by decreasing FZD protein abundance at the membrane. When they are lost, receptor levels increase, thereby increasing signaling. In fact, mutations in these proteins were found to be common in the extremely aggressive pancreatic ductal adenocarcinomas (Jiang et al., 2013). Another way by which Wnt signaling can be increased is by boosting ligand expression: The discovery of Int1 as a murine mammary tumor oncogene, as well as founding the Wnt-signaling field, is a prime example of this (Nusse and Varmus, 1982)(Kahn, 2014).

While activating Wnt signaling is often a driver of tumor initiation, the evolution of the tumor to a fully malignant form seems in some cases to correlate with mutations that shut down the β-catenin dependent Wnt cascade. The prime example for this is melanoma, where increased

β-catenin dependent Wnt signaling actually correlates with better prognosis (Chien et al., 2009). Therefore blocking β-catenin dependent Wnt signaling should not be considered a cure-all and different strategies will have to be applied in different diseases.

Therapeutic Inhibitors of the Wnt pathway

Despite the challenges, especially the pivotal role of the pathway in tissue homeostasis, the Wnt pathway can be therapeutically targeted. An example is the targeting of the bone-derived Wnt inhibitor Sclerostin to treat osteoporosis. The use of a humanized, anti-Sclerostin antibody is currently in phase III clinical trials (Appelman-Dijkstra and Papapoulos, 2016). The approach is successful because of the tissue (bone) specific function of Sclerostin. Discovering and leveraging on the tissue-, disease-specific features is likely the key to the wider application of Wnt pathway modulators. Below we describe different targets and their potential usefulness.

Porcupine: a promising target for efficient Wnt pathway inhibition

One of the most promising avenues for targeting Wnt signaling is to block ligand production. Although, as noted above, many cancers, especially colon carcinomas have activating mutations in components of the Wnt cascade in the receiving cell, there is a growing body of evidence that additional signaling induced by the presence of Wnts is critical to promote tumor progression (Koo et al., 2015) (Lavergne et al., 2011). Currently the best way to interfere with Wnt secretion is inhibition of the acyl-transferase Porcupine (Figure 2).

One such inhibitor -Lgk974- was identified in a high-throughput screen performed on living cells. To achieve this, 2,4 million compounds were tested for their ability to suppress the activity of a transcriptional Wnt reporter in a cell line co-cultured with another cell line overexpressing Wnt3a. Lgk974 binds directly to and inhibits Porcupine (Liu et al., 2013). Currently, it is being tested in a stage 1 dose escalation clinical trial (Lum and Clevers, 2012). Another small-molecule inhibitor of porcupine, ETC-159, has also just entered the phase of clinical trials (Nile and Hannoush, 2016)(Madan et al., 2016).

In mouse tumor models, Porcupine inhibitors (Table 1) showed very promising results in treating various types of cancer. The primary candidates for these studies were cancers

known to be dependent on Wnt secretion, for example due to RNF43 mutations (Liu et al., 2013). Studies were conducted with murine models for mammary carcinomas, basal cell carcinoma, keratoacanthomas, colon cancer, as well as head and neck squamous cell carcinomas (Liu et al., 2013), (Zito et al., 2014), (Larsimont et al., 2015)(Proffitt et al., 2013)(Madan et al., 2016). Another scenario where inhibiting the secretion of ligands might be beneficial is when a cancer exploits them to influence the surrounding tissue to create its own niche.

There are several aspects that need to be considered when evaluating the therapeutic potential of globally blocking the secretion of all Wnts. The first is that systemic abrogation of Wnt secretion will result in defects in gut homeostasis (Valenta et al., 2016). It is therefore a prerogative to either target inhibitors directly to their site of action or then use permissive doses that do not abrogate Wnt signaling to the extent that tissue homeostasis is affected. The existence of a useful therapeutic window is demonstrated by studies showing that treatment with the Porcupine inhibitor Lgk974 resulted in cancer regression but gut homeostasis was unaffected (Liu et al., 2013).

It also needs to be taken into account that blocking Wnt secretion will affect both β-catenin dependent and independent Wnt signaling. The consequences of applying Porcupine inhibitors will therefore depend on what Wnts are present and what pathways are activated. Since β-catenin dependent and independent Wnt signaling seem to influence each other, predicting the outcome is not trivial (Yuzugullu et al., 2009)(Grumolato et al., 2010). An illustrative example is melanoma, where the relative contribution of β-catenin dependent and independent signaling is debated, in particular in later stages such as metastases formation: loss of the β-catenin independent Wnt5a hems tumor growth and metastasis (Weeraratna et al., 2002), (Anastas et al., 2014), however it also seems to lead to activation of β-catenin dependent signaling, which in different studies has positive or negative effects on tumor progression (Yang et al., 2012)(Caramel et al., 2013)(Damsky et al., 2011). While it might seem to restrict the utility of Porcupine inhibitors it may be an advantage to block all Wnt-dependent outputs, β-catenin dependent and independent, and thereby simplify the playing field.

A further critical open question to the therapeutic application of Porcupine inhibitors is its effect on the immune system and how those effects will impinge on the efficacy of treatments. Since the inhibitors are typically applied orally, it cannot be excluded that the loss of Wnt secretion will affect the tumor microenvironment or the proliferation and differentiation of the infiltrating immune cells.

However, the above mentioned challenges behoove further work to understand the consequences of globally blocking Wnt production and find solutions in order to circumvent challenges such as the paramount importance of Wnt signaling for tissue homeostasis.

Alternatives to small molecules are neutralizing antibodies or biologicals to inhibit the receptors

The development of small molecule inhibitors against Wnt pathway components is a challenging task, especially due to the lack of easily targetable enzymes specific for the pathway. An alternative is to use either antibodies against surface molecules like FZD or LRP, or more simply exploit "natural" inhibitors of the cascade.

The difficulty of targeting FZDs with antibodies is the sheer number of them with poorly defined roles in transducing the signal. This raises the problem of the specificity of the antibody to specific receptors as well as possible alternative routes for the cell to transduce the signal if only one specific receptor is blocked. Despite these challenges promising results have been reported with the use of FZD antibodies: one example is the antibody OMP-18R5 that, even though it targets 5 different Frizzleds, appears to specifically hamper tumor growth without affecting normal tissues (Gurney et al., 2012).

Another approach, shown to be promising in mice, is the use of biologically occurring inhibitors. A prime example for this is the injection of SFRP proteins, which are "natural" inhibitors of the pathway (Polesskaya et al., 2003). Instead of using existing SFRPs, there is also the possibility to engineer new ones by simply removing the transmembrane domain of a FZD of interest, thus rendering the Wnt binding part soluble. These engineered proteins can then act as an artificial soluble SFRP (Wei et al., 2011). An additional possibility is to use other soluble inhibitors like DKK (Aicher et al., 2008). Surprisingly, antibodies against DKK1 have anti-tumorigenic effects in cancer cell lines and xenograft models which are

thought to be Wnt-signaling dependent (Sato et al., 2010).. This has to be carefully evaluated, as it points to a broader role for DKK1 in cancer than simply being a negative feedback regulator of the Wnt pathway. With these results in mind it might not be advisable to increase DKK1 dose to inhibit the Wnt pathway, since this might have unexpected effects.

Tankyrase inhibitors

Tankyrase is a member of the PARP [poly (ADP-ribose) polymerases] superfamily of enzymes that add ADP-ribose onto target proteins. With respect to Wnt signaling, Tankyrase PARylates Axin and targets it for proteasomal degradation. Inhibition of Tankyrase thus leads to increased abundance of Axin and consequently to an overactivated destruction complex; the final effect being inhibition of the pathway (Huang et al., 2009).

Initial results with Tankyrase inhibitors seemed to be promising: in particular, the combined administration of AKT, Pi3K and Tankyrase inhibitors to human colon carcinoma cell lines xenografted into mice and rats induced apoptosis in cells escaping the therapy targeting only AKT and Pi3K. This combined therapy was particularly effective in those cases where accumulation of nuclear β-catenin was observed in the tumors. (Arques et al., 2016)

An impasse of this strategy is that Tankyrase has multiple substrates and is critical for many basic cellular processes, e.g. in telomere maintenance, mitosis and insulin mediated glucose uptake; inhibiting it may therefore lead to severe side effects (Riffell et al., 2012).

The β-catenin – TCF interaction is an attractive but elusive target

Another possibility to modulate the β-catenin dependent Wnt signaling cascade very downstream is to target the nuclear function of β-catenin, more specifically by inhibiting the TCF-β-catenin interaction (Valenta et al., 2012). This would be especially efficacious in colon carcinoma, where the majority of the mutations affect the destruction complex. However, there are a number of hurdles that to date have proven insurmountable. First, β-catenin plays important roles in cell adhesion where, in association with E-cadherin, it forms the adherens junctions, and the interaction sites of TCF and E-cadherin overlap. Second, the binding affinity of β-catenin to TCF is quite high (ca. 20 nM). Nevertheless, several screens

have been performed with the aim of disrupting this interaction. Although several compounds were identified that reduced Wnt signaling in reporter assays and inhibit growth of colon cancer cell lines, the mechanisms of action of the molecules remained unclear and their specificity was limited (Kahn, 2014).

However, as mentioned above, β-catenin interacts with various transcriptional co-factors via its C- and N-terminus. Targeting these interactions represents an interesting alternative strategy.

Targeting the interaction between β-catenin and its C-terminal cofactors – a difficult case

Various screens have been conducted in order to find suitable inhibitors of β-catenin's interaction with C-terminal cofactors like CBP and p300. Even though some of these screens yielded efficacious inhibitors, none of them seem to specifically inhibit the interaction with β-catenin. ICG-001, which does inhibit Wnt signaling, generally interferes with CBP's activity and does not inhibit the binding of CBP to β-catenin. Interestingly, ICG-001 does not inhibit the very closely related p300. Since the inhibitor is effective in colon cancer mouse xenograft models, there may be a tissue specific requirement for CBP in the colon (Emami et al., 2004) (McMillan and Kahn, 2005). However, because ICG-001 inhibits CBP, which is part of the general transcriptional machinery, administering this compound could result in severe side effects. Several phase 1 clinical trials are currently being conducted to study the efficacy and side effects of this inhibitor in patients.

Targeting β-catenin's N-terminal interaction partners is the promising alternative

The only known N-terminal binding partners of β-catenin are the paralog proteins Bcl9 and Bcl9l (Bcl9/9l) and indirectly Pygo1/Pygo2 (Figure 1) (Kramps et al., 2002). While in *Drosophila melanogaster* these proteins seem to be mandatory for all Wnt signaling outputs, in the mouse this appears not to be the case and their function seems to be more restricted (Song et al., 2007)(Cantù et al., 2014)(Kramps et al., 2002). For example in the intestinal epithelia, N-terminal co-activators are not needed for normal maintenance of homeostasis,

but only during inflammation-induced regeneration. Moreover colon carcinomas heavily depend on Bcl9/9l to become malignant (Deka et al., 2010). Importantly abrogating the binding between β-catenin and Bcl9/9l has exactly the same effect as the complete deletion of Bcl9/9l; therefore, this genetically very well defined interaction represents an ideal target for the development of small molecule inhibitors (Moor et al., 2015). In terms of specificity, the context-dependent requirement of the N-terminal activators makes it an exciting therapeutic target. Several studies exploring this possibility have been recently published (Hoggard et al., 2015)(Wisniewski et al., 2016).

Also promising seems to be the use of stapled peptides. This technology exploits the fact that the Bcl9-β-catenin interaction is mediated by a helical segment of Bcl9, which binds a large groove of β-catenin's structure. Metabolically stable triazole-stapled Bcl9 □-helical peptides seem to be an efficient approach to inhibit this interaction (Kawamoto et al., 2012) (Takada et al., 2012). These stapled peptides reach a good inhibition in vitro and in mouse xenograft models, but the efficacy of such molecules in the clinic has not yet been tested. A possible drawback of inhibiting Bcl9/Bcl9l functions is suggested by recent findings that show that dysfunctional Bcl9l impairs Caspase 2 expression, thus permitting higher aneuploidy tolerance in colorectal cancer cells (López-García et al., 2017). Whether this is also the case when inhibiting Bcl9l-β-catenin binding will have to be investigated carefully.

Another attractive target is the Bcl9/9l partner, Pygopus 2. From a developmental viewpoint, the requirement for Pygo2 seems to be even more restricted than that of Bcl9/9L: for example, mouse embryos lacking Pygo2 die at E13.5, while Bcl9/9l loss of function is lethal at earlier stages, between E9.5 and E10.5 (Cantù et al., 2014). Pygo1 seems to be negligible, so far no phenotype could be observed upon its loss. Interestingly, Pygo2 plays crucial roles in mammary gland outgrowth as well as in mammary cancer stem cells. Furthermore, it may also play a role in some models of intestinal tumor initiation and progression (Talla and Brembeck, 2016). Additionally, there is evidence that Pygo's chromatin binding ability is required for mammary gland outgrowth (Watanabe et al., 2014). Chromatin binding is not essential for Wnt signal transduction in development and normal homeostasis of mice, suggesting that targeting this interaction will have few side effects (Cantu et al., 2013). Therefore Pygo's chromatin binding capability is a promising target for drug development. The therapeutic potential of targeting the binding of Pygo to Bcl9/9L requires further exploration of when and where this interaction is required; the interaction is relevant also in Wnt-independent contexts (Cantù et al., 2014).

Delivering inhibitors directly to malignant cells via carrier molecules

In the adult organism Wnt signaling is critical for stem cell maintenance and tissue homeostasis, systemically blocking Wnt signaling will therefore be problematic (Valenta et al., 2016). One way of circumventing this is to use the inhibitors at sub-lethal doses, where only the Wnt signaling addicted cancer cells are affected. An alternative is to develop strategies to deliver the inhibitors directly and exclusively to the tumor tissue. This could be done by linking an inhibitor/a toxin to a compound which is attracted by the tumor, as for example was described by Krall and colleagues (Krall et al., 2014)(Wichert et al., 2015) for acetazolamide, a ligand with specific receptors in cancerous lesions in clear-cell renal cell carcinomas. If such molecules also exist in Wnt driven tumors would be needed to be established. Other strategies to deliver the drug via nanoparticles to a specific tissue/cancer have been proposed: one could exploit chemical gradients, such as differences in pH or in oxygen concentration. Tumors are often hypoxic, thus the redox potential in the vicinity of the tumors is altered. Additionally, also liposomes could be used to deliver the inhibitors (Muller and Keck, 2004)(Allen and Cullis, 2013). All these methods have potential, but testing their practical implementation will be an important step in modulating the Wnt-pathway in disease.

Round up and looking forward

Wnt signaling is of paramount importance both in disease and in tissue maintenance; therefore any therapeutic intervention involving Wnt signaling must solve this conundrum. Targeting inhibitors to the afflicted tissue is a promising but underexplored option. Other possible solutions have emerged, and will continue to emerge, as our understanding of the complexities of Wnt signaling in cancer improves.

An exciting target is the Bcl9/9L-Pygo branch of β-catenin dependent Wnt signaling since it is not essential for adult tissue homeostasis but, in the case of colorectal cancer, is required for tumor progression. Blocking the β-catenin-Bcl9/9L interaction is one targetable interface.

Although targeting a protein-protein interaction is challenging, recently, there have been some very promising results using stapled peptides as well as small molecule inhibitors. Further work is needed to determine other cancer types where impinging on the chain of adaptors could be harnessed. Further dissecting the developmental and disease relevance of the chain of adaptors is therefore important. Basic research refining our understanding of intricacies of β-catenin dependent Wnt signaling will reveal other opportunities – identifying additional context and tissue-specific factors is critical.

Another promising avenue is to target Wnt production by inhibiting Porcupine, which is specifically required for the production and secretion of active Wnts. In cancers where Wnt secretion or receptor turnover is over-activated Porcupine inhibition can be effective, e.g. in head and neck squamous cell carcinomas (HNSCC) with Notch mutations (Liu et al., 2013). One future challenge to this is to gauge the consequences of the combined effect of blocking β-catenin dependent and independent Wnt signaling. More work is needed to understand the interplay of these signaling cascades. In tumors with downstream mutations in the Wnt cascade the utility of treatment with Porcupine inhibitors is less obvious; however as mentioned earlier also in such cases ligand mediated augmentation of the signaling likely plays a role in the later stages of tumor progression. Furthermore, β-catenin independent Wnt signaling, which is also blocked by Porcupine inhibition, is known to play a role in late stages of tumorigenesis, for example Wnt5a signaling in melanoma metastases. It is critical to get a better understanding of the biology and the genetics of the tumors that we aim to treat. Given the diversity of the mutational landscapes found in different tumors a therapy which does not work in colorectal cancer or melanoma might well work in mammary tumors.

The practical solution to effectively inhibit Wnt signaling is likely going to be to combine the above-mentioned approaches, and thereby gain a decisive advantage over the tumor. One would need to carefully weigh the impact of a multiplexed approach on homeostasis against the potential enhanced efficacy in killing cancer cells. An additional advantage of a multiplexed strategy would be that it takes away alternative routes for the cancer to escape therapy. Further efficacy from a treatment point of view could also be achieved by using Wnt-signaling inhibition as an adjunct therapy to other molecular medicine approaches, chemotherapy, radiology and/or surgery.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015).

The drug/molecular target nomenclature is in accordance with the BJP concise guide to pharmacology (Alexander et al., 2015).

Competing interests

The authors declare to have no competing interests.

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Table 1: Select Wnt pathway inhibitors and their use in mouse tumor models

compound name	Mode of action	Tested applications	Publications for in vivo inhibitor use if applicable
Lgk974	Inhibits porcupine	Cell lines, div. murine cancer models, phase 1 clinical trial	Liu et al. 2013 Clinical trial identifier: NCT01351103
ETC-159	Inhibits porcupine	Rspo3 translocations in CRC xenografts	Madan et al. 2016
Wnt-C59	Inhibits porcupine	Cell lines, murine cancer models	Proffitt et al. 2013
IWP-2	Inhibits porcupine	Murine keratoacanthoma model, cell lines	Zito et al. 2014
Xav939	Tankyrase 1+2	Cell lines, xenografts	Huang et al. 2009, Arques et al. 2016
ICG-001	Inhibits -catenin-CBP interaction	Diverse murine tumor models	Emami et al. 2004
PRI-724 (2nd generation of ICG-001)	Inhibits -catenin-CBP interaction	Clinical trial phase 1	Clinical trials identifier: NCT01764477, NCT01606579
OMP-18R5 (mAB)	Antibody against FZD receptors	Various xenograft models, clinical trial phase 1	Gurney et al. 2012 Clinical trials identifiers: NCT01973309, NCT01345201
OMP-54F28 (Fzd8-Fc fusion)	competes with Frizzleds for Wnts	Various xenograft models, clinical trial phase 1	Wei et al. 2010 Clinical trial identifier: NCT02092363
FJ9	Inhibits Dishevelled PDZ domain interaction with FZD	Cell lines, xenograft models	Fujii et al. 2007
SAH-BCL9	Inhibits Bcl9- -catenin interaction	Cell lines, xenograft models	Takada et al. 2012
1,4-Dibenzoylpiperazines	Inhibits Bcl9- -catenin interaction	Cell lines	Wisniewski et al. 2016

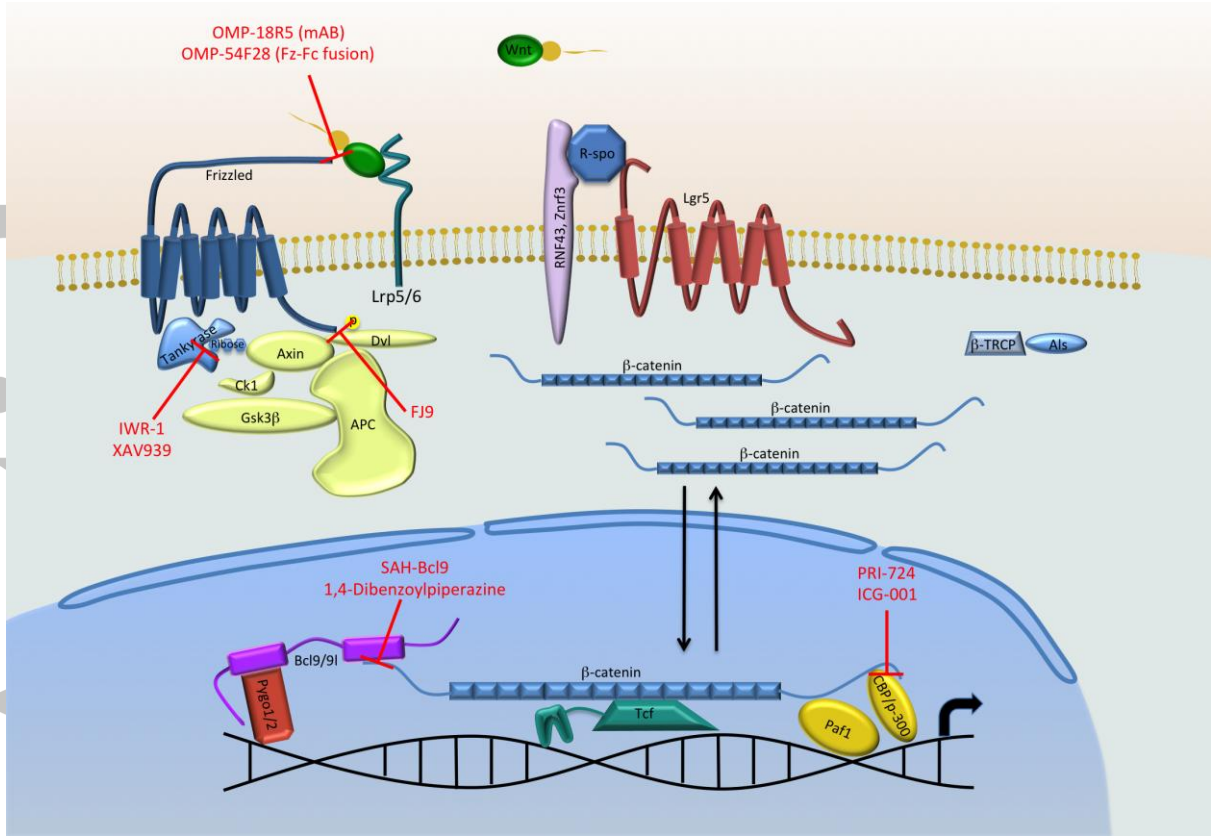
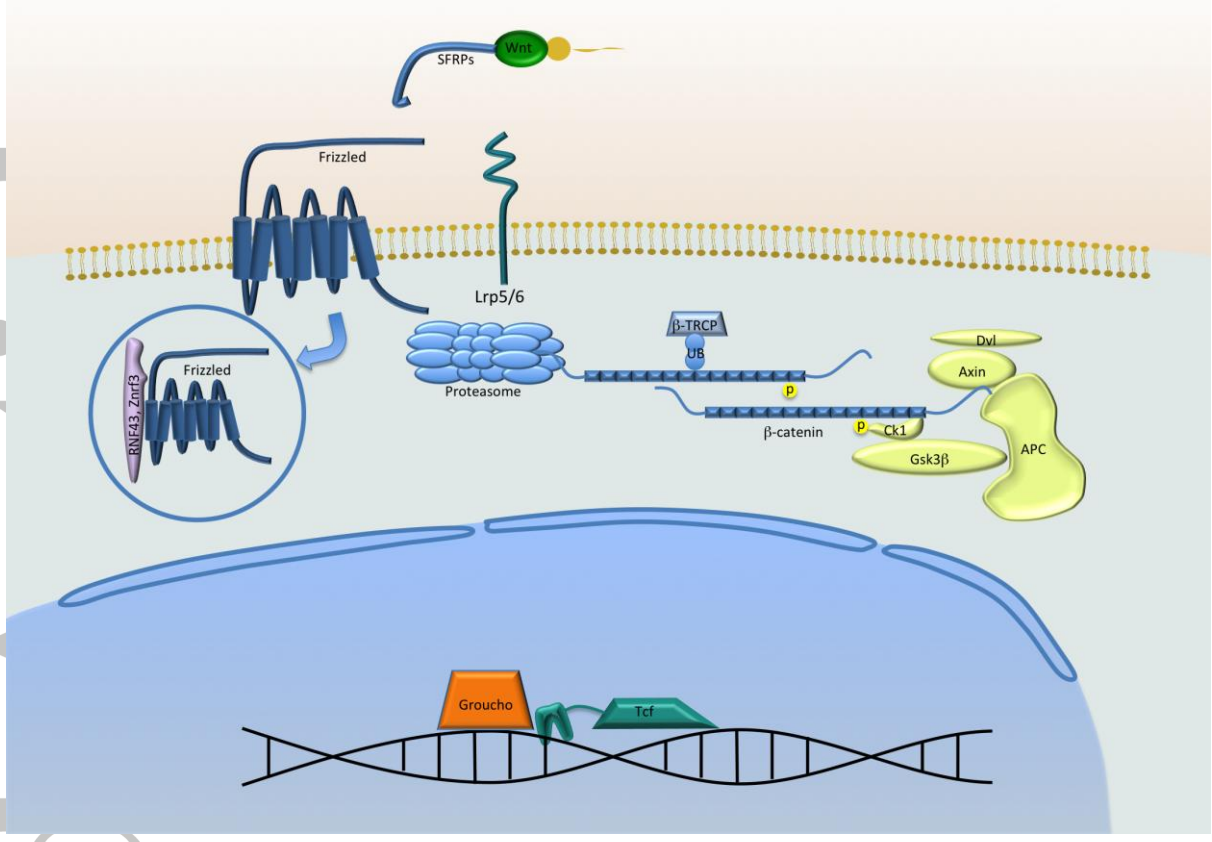


Figure1: The β-catenin dependent Wnt-signaling cascade in the ON-state

a) Upon binding of the Wnts to the receptors of the Frizzled family and the co-receptors LRP5/6, Dishevelled is recruited to the membrane, thus disassembling the destruction complex consisting of AXIN, GSK3β, APC and CK1, preventing phosphorylation and thus protecting β-catenin from proteasomal degradation. This allows β-catenin to accumulate and translocate to the nucleus to initiate target gene transcription. Tankyrases can further increase the signal by marking AXIN for degradation. Furthermore, when ZNRF3 and RNF43 are bound by R-spondin and LGR5 and therefore unable to target Frizzled receptors for degradation Wnt signaling is enhanced in the Wnt-ON state. A further step protecting β-catenin from degradation is the inhibition of E3 ubiquitin ligases such as βTRCP by Armless, at least in *D. melanogaster*.

In red is a selection of Wnt-pathway inhibitors currently used in research. Red bars indicate the interaction they inhibit.



b) The Wnt-signaling cascade in the OFF-state

Without Wnts binding to the FZD and LRP receptors, the destruction complex is active and phosphorylates β-catenin, thus marking it for proteasomal degradation. In the absence of LGR5, a Wnt target gene, also FZD is targeted for degradation by ZNRF3 and RNF43. Furthermore, E3 ubiquitin ligases, like □TRCP, promote proteasomal turnover of β-catenin.

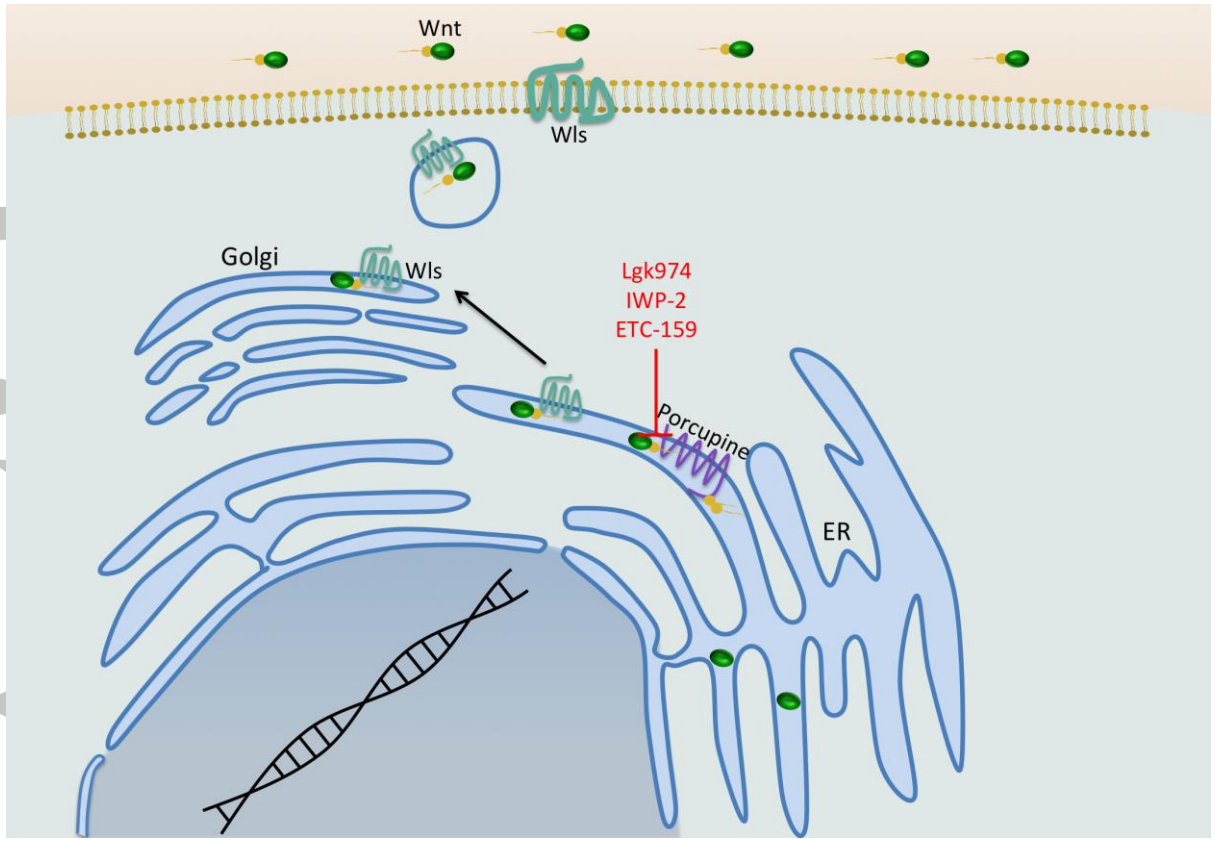


Figure2: The secretory pathway of the ligands of the Wnt pathway

Wnts need to be coupled to fatty acids to be secreted. This happens in the endoplasmatic reticulum (ER) by the acyltransferase Porcupine, which is a prime target for a small molecule inhibitor, since it is the only known enzyme specific to the pathway. Acylation of Wnts allow them to bind to Wntless (Wls) in the Golgi apparatus, which in turn facilitates secretion of the mature Wnts. Wls is transmembrane protein required for the secretion of all Wnts.